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Interaction between unrelated viruses during in vivo co-infection to limit pathology and immunity

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ABSTRACT

Great progress has been made in understanding immunity to viral infection. However, infection can occur in the context of co-infection by unrelated pathogens that modulate immune responses and/or disease. We have studied immunity and disease during co-infection with two unrelated viruses: Ectromelia virus (ECTV) and Lymphocytic Choriomeningitis virus (LCMV). ECTV infection can be a lethal in mice due in part to the blockade of Type I Interferons (IFN-I). We show that ECTV/LCMV co-infection results in decreased ECTV viral load and amelioration of ECTV-induced disease, likely due to IFN-I induction by LCMV, as rescue is not observed in IFN-I receptor deficient mice. However, immune responses to LCMV in ECTV co-infected mice were also lower compared to mice infected with LCMV alone and potentially biased toward effector-memory cell generation. Thus, we provide evidence for bi-directional effects of viral co-infection that modulate disease and immunity.

Introduction

Tremendous progress has been made in our understanding of the requirements for immunity to viral infection (Braciale et al., 2012; Wilson and Brooks 2010). In particular, animal studies utilizing the Lymphocytic Choriomeningitis (LCMV) mouse model of infection have led to the description of numerous fundamental properties of the immune system including the basis for MHC restriction of viral antigens (Zinkernagel and Doherty, 1974, 1975; Doherty and Zinkernagel, 1975), cross-presentation of epitopes by MHC proteins (Storni and Bachmann, 2004), T cell effector mechanisms to control virus infection (Buchmeier et al., 1980; Byrne et al., 1984; Murali-Krishna et al., 1998), the generation and maintenance of T cell memory (Lau et al., 1994; Murali-Krishna et al., 1999; Kaech et al., 2002), and exhaustion of T cell responses during persistent infection (Wherry, 2011; Blattman et al., 2009; Cornberg et al., 2013; Johnson et al., 2011; Wherry et al., 2003b) reviewed in detail by Zhou et al. (2012). Poxviruses have also been widely used to understand how the immune system responds to infection (Miller et al., 2008) and are currently being investigated for use as potential vaccine vectors (Tartaglia et al., 1990) for many important human pathogens such as HIV (Haynes et al., 2014).

Despite this progress, an important caveat to such illuminating laboratory experiments is that they are almost always done in isolation, under specific pathogen-free (SPF) conditions, whereas most "real-world" infections likely occur in the context of co-infection by unrelated pathogens that have the potential to modulate immune responses and/or alter disease (Stelekati and Wherry, 2012). Current studies suggest co-infection with different pathogens is a common occurrence that can alter the progression of disease (Stelekati and Wherry, 2012; Seki et al., 2004; Stoicov et al., 2004; Walzl et al., 2000; Furze et al., 2006). One early example of such interaction is exacerbation of Listeriosis in mice to lethal disease during co-infection with LCMV (O'Connell et al., 2004; Navarini et al., 2006). Recent studies have also shown that enteric bacterial strains promote infection by poliovirus via mucosal routes (Kuss et al., 2011). Another potential consequence of viral co-infection is that one virus may supply ancillary functions or suppress immune functions for another (Sharp and Simmonds, 2011). Such a relationship has previously been described for Hepatitis B (HBV) and Hepatitis D (HDV) viruses: HDV cannot form mature virions without the presence of the Hepatitis B structural proteins (Bonino et al., 1986). Co-infection of HBV and HDV or super infection of persistently infected HBV patients with HDV can also alter disease progression, by increasing liver pathology resulting in poorer prognosis in patients due to altered immune responses and type I interferon signaling in the host (Abbas and Afzal, 2013; Alvarado-Mora et al., 2013).

In order to understand the complex relationships that may be occuring during virus co-infection, we have studied co-infection of mice with ectromelia virus (ECTV) and LCMV, two unrelated viruses that are both endemic to mice. Although previous studies of co-infection with the related vaccinia virus (VACV) and LCMV have shown no alteration in either LCMV CD8 T cell responses or disease, such studies may minimize the role of these interactions

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(Brenan and Zinkernagel, 1983; Valentine et al., 2012), as VACV is not endemic to mice, and many VACV immune evasion proteins do not function well in mice. ECTV is a DNA virus of the orthopoxvirus family and encodes a number of proteins that block the production of and signaling by Type I interferons (IFN-I) (Smith and Alcami, 2002; Karupiah et al., 1993). Experimental inoculation of mice with ECTV typically results in a lethal infection in mice due to high virus replication in the liver and acute hepatic disease (Chapman et al., 2010; Jacoby and Bhatt, 1987). Conversely, LCMV infection of mice results in the rapid expansion of virus-specific CD8 T cells that limit viremia with viral control and clearance within 7 days post infection (Wherry et al., 2003b; Oldstone and Dixon, 1968). In striking contrast to ECTV, LCMV induces robust Type I IFN production in mice with peak production during the first 12–48 h of infection (Zhou et al., 2010; Teijaro et al., 2013). Furthermore, CD8 T cell responses to LCMV in mice are highly dependent on IFN-I signaling for sustained expansion (Aichele et al., 2006; Kolumam et al., 2005). Infection of Interferon receptor deficient ($IFNAR^{-/-}$) mice with LCMV results in a defective CD8 T cell response that is unable to control infection (Ou et al., 2001). Therefore, we hypothesize that co-infection with ECTV and LCMV has the potential for bi-directional effects on disease and immunity by IFN-I suppression of ECTV replication and disease while limiting LCMV-specific CD8 T cell responses.

We show here that ECTV/LCMV co-infection of mice results in decreased ECTV viral load and ameliorates ECTV-induced disease. Furthermore, we show that this effect is likely due to IFN-I induction by LCMV that is able to overwhelm ECTV mechanisms for suppression of IFN-I production and signaling. Conversely, we also show that ECTV partial suppression of IFN-I production during co-infection with LCMV results in diminished CD8 T cell responses to LCMV. Additionally, the LCMV response is biased towards the formation of memory CD8 T cells with a TNF-deficient effector-memory phenotype that has been shown to be less protective in other studies (Wherry et al., 2003a; Zaph et al., 2004; Bachmann et al., 2005). Thus, we provide the first experimental evidence for bi-directional effects of these two unrelated viruses during co-infection to modulate disease and immunity. These findings likely have implications for disease and transmission of these viruses in wild mouse populations, but more importantly suggest that differences in immune responses during viral infection may be in part due to heterologous virus infection (Oldstone, 2002; Childs et al., 1992).

Materials and methods

Mice

6–8 week old female C57Bl/6J mice were purchased from Jackson laboratories (Bar Harbor, Maine). IFNAR1-deficient mice ($IFNAR^{-/-}$) were purchased from Jackson laboratories (Bar Harbor, Maine) and bred in our ASU animal facilities. All studies were conducted according to animal protocol 12-1229R under the approval and guidance of the Arizona State University Institute for Animal Care and Use Committee.

Cells and viruses

BHK cells were maintained in complete Eagle's MEM (5% fetal bovine serum (FBS), 2 mM L-glutamine (L-Q), 100 U/mL penicillin, 100 μg/ml streptomycin). Vero and MC57 cells were maintained in complete DMEM (10% FBS, 2 mM L-Q, 100 U/ml penicillin, 100 μg/mL streptomycin). LCMV Armstrong and LCMV clone-13 stocks were kindly provided by Rafi Ahmed (Emory University, Atlanta GA) and produced in BHK cells as previously described (Welsh and Seedhom, 2008). The titer of LCMV stocks and mouse serum samples were determined by plaque assay on Vero cell monolayers as previously described (Hersperger et al., 2012). ECTV expressing the β-gal gene in the CHO locus (US17-βgal) was a gift from Dr. Mark Buller (St Louis University, St Louis MO). ECTV stocks were propagated in Vero cells as previously described (Alejo et al., 2009). ECTV titers in mouse liver homogenates were determined on VERO cell monolayers. Briefly, liver samples were weighed and homogenized in PBS to 10% w/v. Vero monolayers were infected following three freeze-thaw cycles for 1 h prior to overlay with a 1:1 ratio of 1% agarose and 2 × -MEM supplemented with 10% fetal bovine serum. After three days at 37 °C, the second overlay of a 1:1 ratio of 1% Agarose and 2 × -MEM supplemented with 10% fetal bovine serum and X-gal (20 mg/ml) was applied. Four days after the secondary overlay blue ECTV plaques were counted.

In vivo infections

Unless otherwise stated, LCMV and ECTV stocks were diluted to 10^6 pfu/ml in 1 × PBS prior to intraperitoneal infection in a volume of 100 ul, delivering a total of 10^5 pfu per mouse. Unless otherwise indicated, co-infected mice received ECTV immediately followed by LCMV inoculation. Mice were monitored daily for clinical disease (hunched posture, ruffled fur, non-motility) and euthanized in accordance with our approved IACUC protocol when terminal diseases symptoms were observed at the indicated times post infection.

Peptides

Lymphocytic choriomeningitis virus CD8 T cell epitopes GP33 ($H\text{-}2D^b$, KAVYNFATC) and NP396 ($H\text{-}2D^b$, FQPQNGQFI) were purchased from Genscript (Piscataway, NJ).

Cell surface antibody staining

Single cell suspensions were prepared from splenocytes as previously described (Murali-Krishna et al., 1998). Erythrocytes were lysed with ammonium chloride lysis (ACK) buffer purchased from Lonza (Allendale, NJ) and FACS staining was done as previously described (Murali-Krishna et al., 1998) in 96 well plates with fluorochrome-labeled monoclonal antibodies: anti-CD8 (clone 53-6.7), anti-CD44 (clone IM7), anti-PD-1 (clone J43), anti-CD4(clone GK1.5) and anti-CD62L (clone MEL-14) or APC labeled GP33-tetramer (Wherry et al., 2003b). Samples were then fixed in 1% paraformaldehyde solution and immediately acquired on a BD LSR II Fortessa flow cytometer (San Jose, CA) and analyzed using FlowJo software (Tree-Star, Ashland, OR). All surface monoclonal antibodies were purchased from BD Pharmigen (San Diego, CA) or eBioscience (San Diego, CA).

Intracellular cytokine staining

For quantitation of ECTV-specific T cell responses, splenocytes (10^6/well) were stimulated with uninfected MC57 cells or with ECTV infected MC57 cells (MOI:1, at 24 h post infection) as previously described (Hersperger et al., 2012). For quantitation of LCMV-specific T cell responses, splenocytes were cultured alone or with 1 μM LCMV peptide epitopes as previously described (Murali-Krishna et al., 1998). After 5 h of stimulation, cells were permeabilized and intracellular cytokine producing cells detected by staining with anti-IFNγ (clone XMG1.2) and anti-TNF (clone MP6-XT22) antibodies purchased from BD Pharmigen (San Diego, CA) or eBioscience (San Diego, CA). The samples were acquired and analyzed as described above.

Interferon-I ELISA

Mouse interferon beta and interferon alpha ELISA kits were purchased from PBL Assay Science (Piscataway, NJ) and used

according to manufacturer's instructions to detect serum Type I IFN levels.

Histology

Formalin fixed liver sections were cut into 20 μm thick tissue sections on a microtome. Liver samples were stained with x-gal (20 mg/mL) using the β-galactosidase reporter gene staining kit purchased from Sigma (St Louis, MO) following the manufacturer's instructions. Adjacent sections of formalin fixed liver tissue were stained with hematoxylin and eosin as previously described (Fischer et al., 2008). 20x images were taken with a Zeiss Axioskop (Thornwood, NJ) and evaluated for pathology and punctate blue x-gal staining.

Statistics

Prism software (Graphpad, La Jolla, CA) was used to calculate t-test p values to determine significance or log-rank test to determine survival curve significance ($^{*}=p\leq 0.05$, $^{**}=p\leq 0.01$, $^{***}=p\leq 0.001$).

Results

ECTV/LCMV co-infection reduces ECTV disease and viral load

We first determined if ECTV/LCMV co-infection alters disease in mice compared to ECTV infection alone, by monitoring physical symptoms and mouse survival. As expected, mice infected with ECTV alone exhibited extreme disease symptoms starting at 5 days post infection and required euthanasia by 7 days post infection (Fig. 1A). Surprisingly, 6 week-old ECTV/LCMV co-infected mice exhibited a delay in ECTV-induced disease, with survival between 12 and 17 days post infection, more than twice that observed in mice infected with ECTV alone. As Orthopoxvirus infections have been shown to be age and immune status dependent, with older mice typically having reduced poxvirus replication and less severe disease (Esteban and Buller, 2005), we also compared mice infected with ECTV alone to ECTV/LCMV co-infection in 8-week old mice. We observed no difference in ECTV disease in older mice compared to younger mice, presumably due to the high dose of inoculation used, since all mice infected with ECTV alone required euthanasia by 7 days post-infection. In striking contrast, ~70% of 8 week-old ECTV/LCMV co-infected mice exhibited long-term disease-free survival. Overall, ECTV/LCMV co-infection resulted in 50% survival of mice, with no detectable ECTV in liver homogenates at >60 days post infection compared to 0% survival after mice infected with ECTV alone. Thus, these results demonstrate that ECTV/LCMV co-infection can ameliorate ECTV disease in mice.

ECTV induced mortality in mice is known to correlate with high viral titers in the liver that result in in acute hepatic failure (Esteban and Buller, 2005; Xu et al., 2012). In order to determine whether the delay in and/or amelioration of ECTV-induced disease in ECTV/LCMV co-infected mice was due to suppression of ECTV viral loads, we next measured ECTV viral titers in the liver of ECTV/LCMV co-infected mice compared to mice infected with ECTV alone. Mice infected with ECTV alone exhibited high liver viral titers, with up to 10^9 pfu/gram by 6 days post-infection (Fig. 1B). However, ECTV/LCMV co-infected mice exhibited a ~1000 to 100-fold decrease in ECTV liver viral load, remaining below 10^7 pfu/gram up to 9 days post infection. Thereafter, ECTV/LCMV co-infected mice were either able to completely control ECTV infection or succumbed to lethal disease. Notably, in analysis of liver tissue sections directly *ex vivo*, mice infected with ECTV alone displayed punctate x-gal staining across the entire liver section, whereas x-gal staining was undetectable in ECTV/LCMV co-infected mice at day 5 post infection (Fig. 1C, top panel). These findings were also consistent with histological staining (H&E stain) in which liver tissues at 5 days post infection from mice infected with ECTV alone showed multiple necrotic lesions, whereas liver sections from ECTV/LCMV co-infected mice did not exhibit any detectable necrotic lesions (Fig. 1C, bottom panel). Taken together, these results show that ECTV/LCMV co-infection reduces ECTV virus load in the liver and therefore ameliorates disease.

In order to investigate if the reduced disease in ECTV/LCMV co-infected mice was due to LCMV prevention of initial ECTV infection of cells, versus suppression of ECTV replication, we tested whether infection of mice with decreasing doses of ECTV during LCMV co-infection had a similar effect on disease and viral loads. We reasoned that if LCMV inhibited initial seeding of ECTV in the liver we would expect mice receiving lower doses of ECTV to also have less disease and enhanced survival in the absence of LCMV infection. Mice that received up to 1000-fold lower doses of ECTV exhibited similar disease at 5 days post-infection compared to the high dose inoculation used above, and 100% of these mice required euthanasia by 7 days post infection. Surprisingly, lower inoculum doses of ECTV during ECTV/LCMV co-infection had no impact on suppression of disease: ECTV/LCMV co-infected mice at all doses of ECTV demonstrated delayed disease until day 16 post infection and had no statistical difference in the percentage of mice that survived long-term, similar to that observed during high dose infection (Fig. 1D). These data are consistent with the hypothesis that the observed suppression of ECTV disease in ECTV/LCMV co-infected mice is not due to competition for target cells or blockade of initial ECTV seeding in the liver, but rather suppression of ECTV replication or infection after initial seeding.

The timing of LCMV infection is crucial for reducing disease during ECTV co-infection

We next hypothesized that the observed reduction in ECTV replication and disease during LCMV co-infection may be dependent on the timing (prior exposure or post exposure) of LCMV infection due to the potential for enhancement of early innate events by LCMV to suppress ECTV infection. Infection with LCMV up to 2 days prior to ECTV infection resulted in similarly reduced ECTV viral loads and disease as observed during concurrent ECTV/LCMV co-infection (Fig. 2A). Mice infected with LCMV 3 days prior to ECTV infection demonstrated a minor delay in disease progression, with mice surviving on average 3 days longer than mice infected with ECTV alone. In striking contrast, mice inoculated with LCMV 1 or 2 days post ECTV infection showed no reduction in ECTV viral loads or disease compared to mice infected with ECTV alone, with 100% of these mice exhibiting lethal disease symptoms by 7 days post-infection (Fig. 2B). Thus, the timing of co-infection for the suppression of ECTV replication and disease suggests that the effects of LCMV on ECTV are to enhance early innate events to suppress ECTV viral infection in the liver.

ECTV/LCMV co-infection nullifies ECTV abrogation of IFN-I production

Because LCMV is known to induce potent Type I Interferon responses (Teijaro et al., 2013), and ECTV is sensitive to Type I Interferons (Karupiah et al., 1993; Xu et al., 2012), we next hypothesized that the early innate immune suppression of ECTV could be due to an overwhelming IFN-I response. This would be consistent with the observation that LCMV inoculation 1–2 days prior to ECTV infection resulted in similar reduction in disease and ECTV liver titers, as Type I IFN production is maximal at 1–2 days post LCMV infection and is curtailed thereafter (Zhou et al., 2010). Serum levels of total IFNα and IFNβ in ECTV infected mice were undetectable, as expected, presumably due to the ability of ECTV viral proteins to completely shut down IFN-I production (Esteban and Buller, 2005). In contrast, mice infected with LCMV alone induced potent IFN-I responses, with >12,000 pg/mL total IFNα and >500 pg/mL IFNβ in the serum of mice infected with LCMV alone by 24 h post infection. Thereafter, IFNα and IFNβ levels decreased to 9000 pg/mL and 100 pg/mL respectively by 48 hours

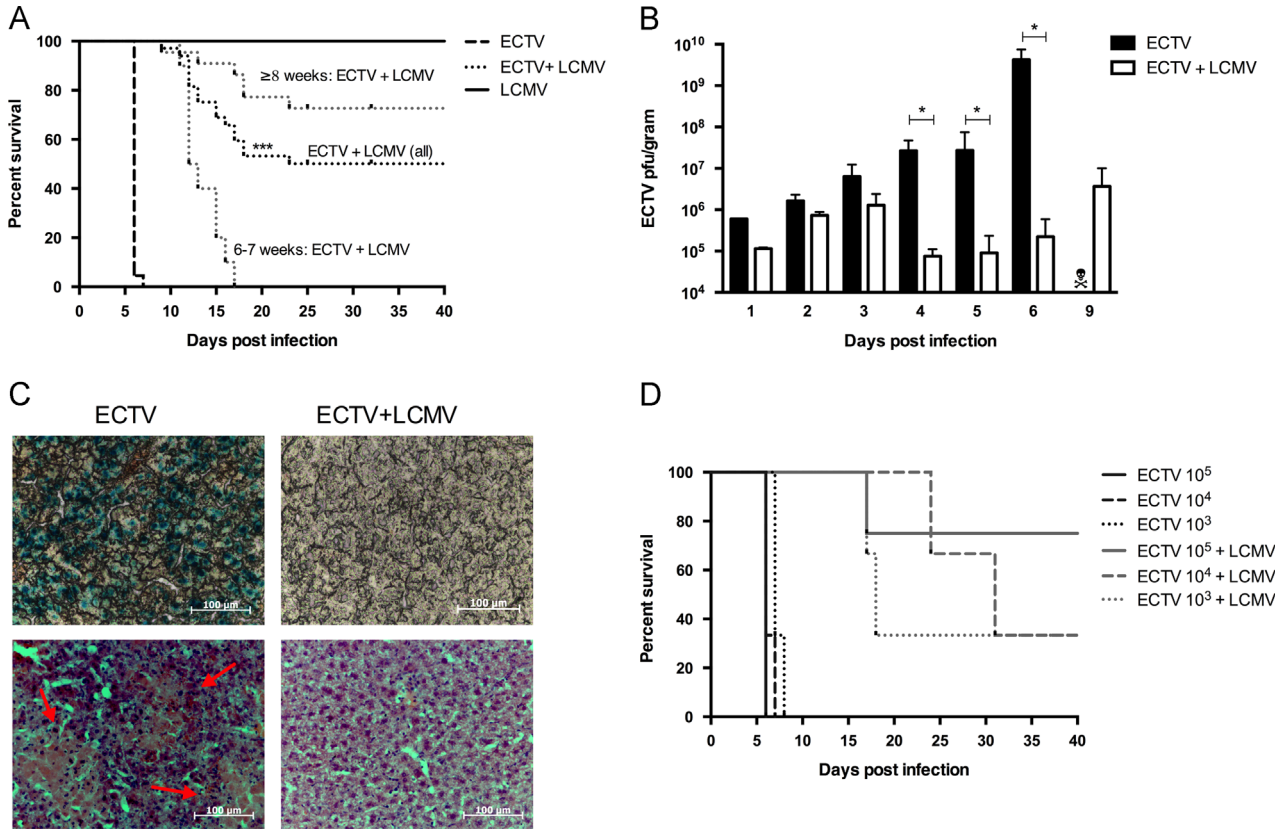

Fig. 1. ECTV/LCMV co-infection reduces ECTV disease and viral load. 6-8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. All mice were monitored daily for morbidity and mortality. [A] Survival of ECTV infected ($n=34$), LCMV infected ($n=20$) and ECTV/LCMV co-infected ($n=47$) 6–8 week-old mice. [B] Mean ECTV viral load (pfu/gram) in liver in ECTV infected and ECTV/LCMV co-infected mice on days indicated post infection ($n=3$–5 mice/group). [C] Top panel: 20X view of formalin fixed liver section stained with X-gal at day 5 post infection. Bottom panel: Histological stain (H&E) of formalin fixed liver sections at day 5 post infection, red arrows point to necrotic lesions. [D] Survival of 8-week old female C57Bl/6 mice injected with 10^5, 10^4 or 10^3 pfu ECTV-US17-βgal ip. Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip ($n=3$–5 mice/group).

in mice infected with LCMV alone and were low to undetectable thereafter (Fig. 3A and B). Although ECTV/LCMV co-infected mice also exhibited an increase in serum levels of both IFNα and IFNβ, compared to mice infected with ECTV alone, IFNβ levels were lower in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone. IFNα levels were not statistically different between LCMV and co-infected animals. Thus, these results suggest that although ECTV is able to partially attenuate IFN-I production during LCMV co-infection, sufficient IFN-I levels remain to suppress ECTV replication and ameliorate disease.

In order to directly test the role of IFN-I in suppression of ECTV replication and disease during ECTV/LCMV co-infection, we tested whether ECTV/LCMV co-infection could rescue IFNAR$^{-/-}$ mice from disease. Mice infected with ECTV alone and ECTV/LCMV co-infected mice had indistinguishable ECTV viral loads and exhibited similar disease, requiring euthanasia by 7 days post-infection (Fig. 3C). Thus, LCMV co-infection does not rescue IFNAR$^{-/-}$ mice from lethal ECTV disease. These results suggest that Type I IFN signaling is the main mechanism for LCMV suppression of ECTV replication and disease.

LCMV co-infection does not significantly enhance ECTV-specific CD8 T cell responses

Resistance to mousepox has been shown to correlate with rapid, more enhanced cytotoxic responses (Jacoby and Bhatt, 1987; Chaudhri et al., 2004). Therefore, an alternative explanation for the observed decrease in ECTV disease during LCMV co-infection is that LCMV alters ECTV-specific adaptive immune responses, and thus attenuates immunopathology. It should be noted that although no studies have shown a dependence on Type I IFN for induction of ECTV T cell responses, as has been shown with LCMV, other Orthopoxviruses do not require Type I IFN for induction of CD8 T cell immunity (Brenan and Zinkernagel, 1983). We observed no difference in the ECTV-specific CD8 T cell response between mice infected with ECTV alone or ECTV/LCMV co-infected mice at 5 days post infection when disease symptoms became apparent (Fig. 4A and B). Moreover, in both groups of mice the CD8 T cells had equivalent production of IFNγ and TNF (data not shown). Thus, LCMV co-infection does not appear to alter initial ECTV CD8 T cell responses, or immunopathology resulting from these cells, but rather the observed effects of co-infection on reduction of ECTV replication and amelioration of disease are likely limited to alteration of innate immune production of IFN-I.

Decreased IFN-I during ECTV/LCMV co-infection limits LCMV-specific CD8 T cell expansion

In addition to the effect of LCMV co-infection to reduce ECTV replication and disease, we next hypothesized that production of ECTV proteins that suppress Type I IFN production (Smith and Alcami, 2002) during LCMV co-infection may impair the generation of LCMV-specific CD8 T cell responses that are critically dependent on Type-I IFN for sustained proliferation (Aichele et al., 2006; Kolumam et al., 2005). Peak LCMV CD8 T cell responses to the immunodominant GP33 and NP396 epitopes (Wherry et al., 2003b) at 9 days post-infection (Fig. 5**A**) in mice infected LCMV alone were $>35\%$ of the total CD8 T cell population with on average $>6\times10^6$ LCMV-specific cells. However, ECTV/LCMV co-infection decreased the peak LCMV response by 2–3 fold as GP33 and NP396 epitope-specific CD8 T cells comprised

$<20\%$ of the total CD8 T cell population with on average $<2\times10^6$ LCMV-specific CD8 T cells (Fig. 5B). This effect was not due to differences in the timing of expansion of the LCMV-specific CD8 T cells, as a similar reduction in the effector T cell response was also seen at 7 and 9 days post ECTV/LCMV-infection (Fig. 5C). Moreover, and consistent with previous reports that show that Type I IFN are necessary for sustained proliferation of CD8 T cells but not the initial activation and early proliferation of these cells (Kolumam et al., 2005), we observed no difference in the CD8 T cell response to LCMV between mice infected with LCMV alone and ECTV/LCMV co-infected mice at 5 days post-infection (Fig. 5C). Thus, incomplete ECTV suppression of Type I IFN during LCMV co-infection results in reduced LCMV-specific CD8 T cell expansion.

In order to determine if the route of infection of ECTV can impact the resulting decrease in T cell immunity, LCMV-specific CD8 T cell responses were measured during co-infection of ECTV infection via footpad injection, which mimics the presumed natural route of ECTV through skin abrasion. The number of LCMV-specific CD8 T cells in the spleen was measured by IFNγ production after stimulation with GP33 and NP396 epitopes at day 9 post infection. Consistent with previous results, co-infection with ECTV resulted in a statistically significant, greater than 2-fold, decrease of LCMV effector CD8 T cells at the peak of the response (Fig. 5D). Indicating the route of infection does not alter the potential for immune modulation during co-infection.

Additionally, although not statistically significant with the number of mice used in these studies, the percentage of LCMV-specific memory CD8 T cells in ECTV/LCMV co-infected mice was consistently lower than that observed in mice infected with LCMV alone at >35 days post infection. Thus, memory CD8 T cell populations specific for the immunodominant NP396 and GP33 epitopes appeared to be proportional to peak effector T cell responses in both groups. (Fig. 6A).

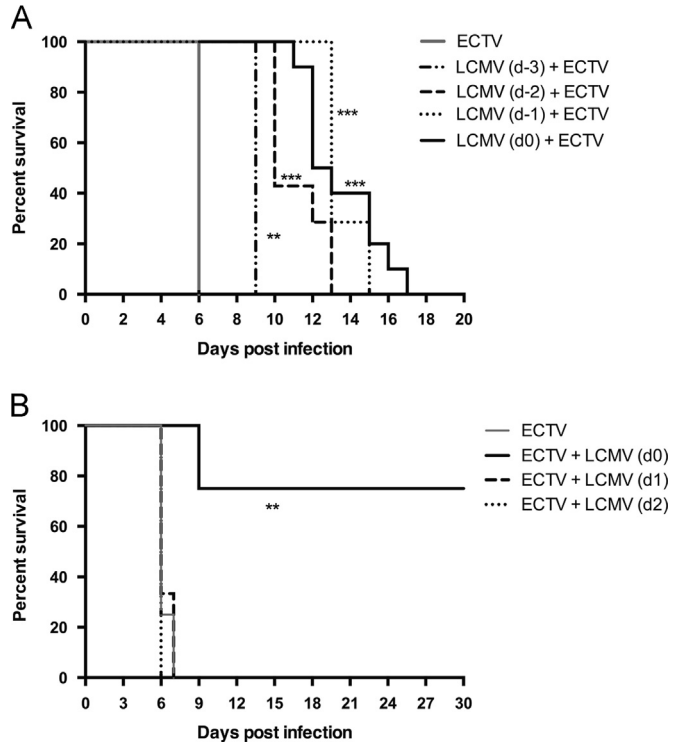

Fig. 2. The timing of LCMV infection is crucial for reducing disease during ECTV co-infection. [A] Survival of 6 week-old mice infected with 10^5 LCMV Armstrong (ip) 1, 2 or 3 days prior to ECTV-US17-βgal (ip) infection. [B] Survival of ECTV-US17-βgal infected 8-week old mice (d0) that received LCMV Armstrong immunization 1 or 2 days post ECTV exposure ($n=3$–5 mice/group).

Decreased CD8 T cell responses to LCMV during ECTV/LCMV co-infection do not impair control of LCMV or T cell memory function

We next asked whether the observed reduction in LCMV-specific CD8 T cell responses during ECTV/LCMV co-infection impaired immune control of LCMV. Surprisingly, we observed no

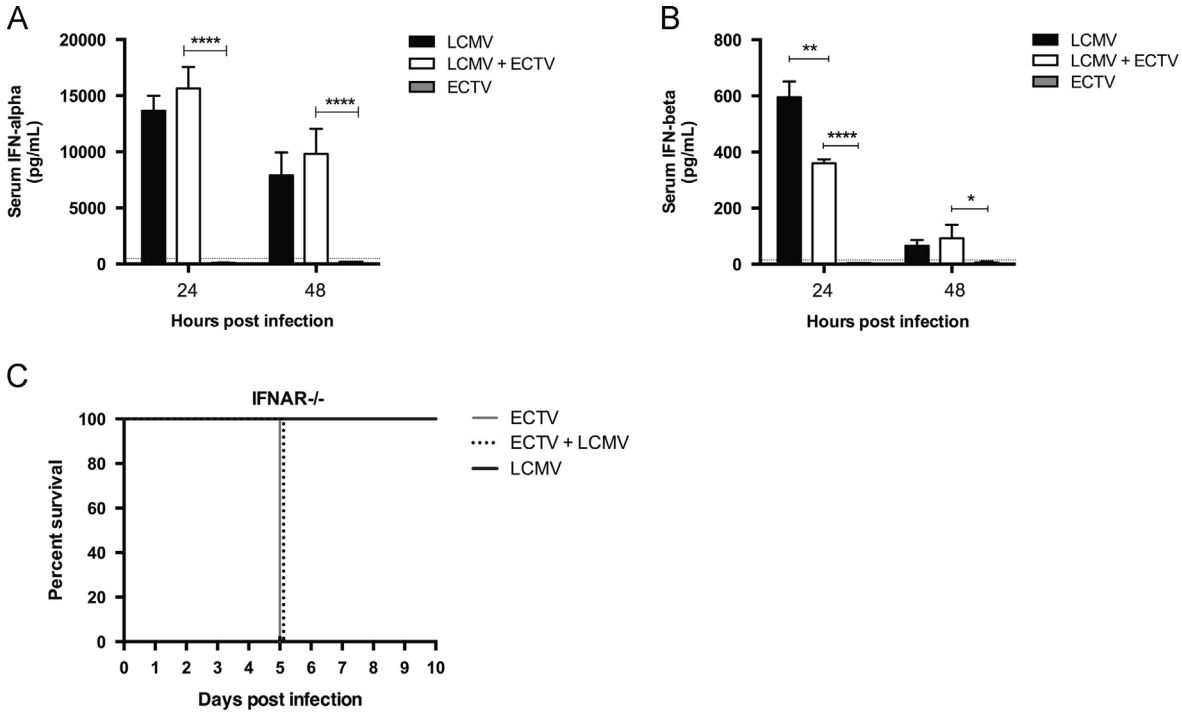

Fig. 3. ECTV/LCMV co-infection nullifies ECTV abrogation of IFN-I production. 6–8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. [A] IFN-alpha levels in serum of ECTV/LCMV co-infected mice as compared to LCMV only and ECTV only control mice at 24 and 48 h post infection ($n=3$–5 mice/group). [B] IFN-beta levels in serum of ECTV/LCMV co-infected mice as compared to LCMV only and ECTV only control mice at 24 and 48 h post infection ($n=3$–5 mice/group). **[C]** Survival of 6–8 week old female IFNAR$^{-/-}$ mice injected with 10^5 pfu ECTV-US17-βgal ip (100 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip ($n=3$–5 mice/group).

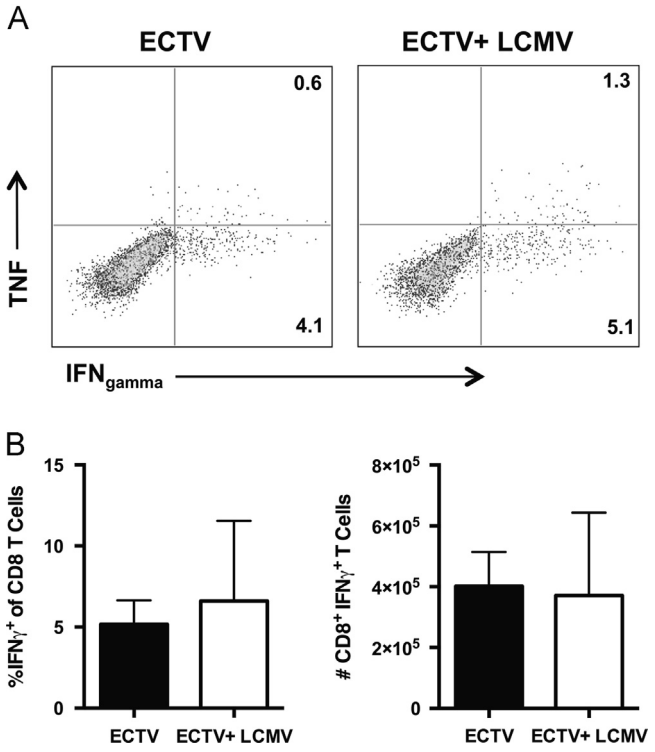

Fig. 4. ECTV/LCMV co-infection does not significantly enhance magnitude of ECTV-specific CD8 T cell response. 6–8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV Armstrong ip. At the indicated time post infection, splenocytes were harvested, processed to single cell suspensions and stimulated for 6 h with ECTV infected MC57 cells prior to intracellular cytokine staining. [A] Representative FACS plots of the ECTV-specific CD8 T cell response at day 5 post infection in the spleen from ECTV infected and ECTV/LCMV co-infected mice. [B] Mean percentage of (left panel) and total number (right panel) of ECTV-specific CD8 T cells in the spleen on day 5 post infection as measured by IFNγ production ($n=3$–5 mice/group).

difference in serum LCMV titers at 7 days post-infection between mice infected with LCMV alone and ECTV/LCMV co-infected mice, with neither group having detectable LCMV levels at this time. However, we cannot rule out that there were small differences in the kinetics of control of LCMV. Many studies have suggested that the strength or duration of the initial stimulus has a dramatic impact on the generation and function of CD8 T cell memory (Kaech et al., 2002; Wherry et al., 2003b; Ou et al., 2001). Although we observed no impact of ECTV/LCMV co-infection on CD8 T cell mediated control of LCMV, we reasoned that if the diminished CD8 T cell responses in ECTV/LCMV co-infected mice resulted in slight impairment of LCMV-specific immunity, then this might alter the bias generation of LCMV-specific memory T cells or their function.

To test whether LCMV-specific memory CD8 T cells in ECTV/LCMV co-infected mice were impaired in their effector functions we next measured the ability of memory cells from mice infected with LCMV alone or ECTV/LCMV co-infected mice to produce the key cytokines IFNγ and TNF. We observed a decrease in the relative proportions of memory cells, specific for NP396 and GP33 epitopes, that were positive for both IFNγ and TNF: 89% of CD8 T cells producing IFNγ in response to these epitopes in mice infected with LCMV alone were double-positive for TNF while only 41% IFNγ positive LCMV-specific CD8 T cells in ECTV/LCMV co-infected mice also produced TNF (Fig. 6B). Moreover, the number of central-memory ($CD62L^{high}$) CD8 T cells specific for GP33 was decreased three-fold in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone (Fig. 6**C**). Taken together, these results suggest that CD8 T cell control of LCMV is partially impaired in ECTV co-infected mice resulting in less functional memory cells that may be biased toward an effector-memory ($CD62L^{low}$) phenotype.

Finally, we tested whether the reduced cytokine production and effector-memory bias of CD8 T cell populations in ECTV/LCMV co-infected mice impacted the ability of these mice to control subsequent LCMV infection. We challenged ECTV/LCMV co-infected or mice infected with LCMV alone at memory time points (60 days post-infection) with the virulent clone-13 strain of LCMV (Sullivan et al., 2011; Ahmed et al., 1984; Smelt et al., 2001). Both groups of mice, whether previously infected with LCMV alone or with ECTV/LCMV, and challenged with LCMV clone-13 at 60 days post-infection, had undetectable LCMV viral titers in the serum at 7 days post-challenge (Fig. 6D). In contrast, non-immunized mice infected with LCMV clone-13 were unable to control LCMV replication and had high viremia ($\sim 10^5$ pfu/ml) in the serum at 7 days post-infection. NP396 or GP33 specific memory CD8 T cells from either ECTV/LCMV co-infected or mice infected with LCMV alone expressed low levels of PD-1, an inhibitory protein associated with decreased CD8 T cell function (Blattman et al., 2009), after LCMV clone-13 challenge (Fig. 6E). Thus, although ECTV co-infection reduced the generation and function of memory LCMV-specific CD8 T cell populations, in addition to biasing these cells to an effector-memory phenotype which has been shown to be less protective during LCMV challenge (Wherry et al., 2003a; Bachmann et al., 2005), sufficient memory T cells persisted in both groups to mount protective responses to LCMV clone-13 challenge.

Discussion

Our results demonstrate interaction between endemic but unrelated viruses during co-infection of mice to limit disease and immunity. Mice co-infected with LCMV and ECTV demonstrated reduced ECTV replication resulting in reduced disease and enhanced survival. Conversely, ECTV co-infection resulted in reduced LCMV-specific CD8 T cell responses. The data suggest that these effects are primarily due to modulation of Type I IFN levels, with primarily lower IFNβ levels in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone and no difference in disease progression during ECTV/LCMV co-infection in $IFNAR^{-/-}$ mice compared to mice infected with ECTV alone. This is further supported by more striking amelioration of disease in ECTV/LCMV co-infected older mice in which Type I IFN signaling has previously been shown to limit Orthopoxvirus replication (Xu et al., 2012).

Our results implicate Type I IFN production and/or signaling as the main mechanism by which LCMV suppresses ECTV replication and disease in ECTV/LCMV co-infected mice. The kinetics of LCMV infection and induction of Type I IFN responses in this model appears to be critically important as co-infection with LCMV 3 days prior had minimal impact on ECTV disease while inoculation with LCMV >1 day after ECTV infection showed no effect. We suggest in the former case LCMV-induced Type I IFN production is curtailed after 3 days to levels that are effectively blocked by ECTV proteins. In contrast, LCMV infection after establishment of ECTV infection is likely ineffective as ECTV proteins have effectively shut down Type I IFN signaling (Esteban and Buller, 2005; Brownstein et al., 1993). Our results also show that ECTV modulation of Type I IFN production during ECTV/LCMV co-infection attenuates LCMV-specific CD8 T cell responses that are dependent on direct signaling via Type I IFN for sustained proliferation. It is unlikely in this context that ECTV is modulating Type I IFN intracellular signaling as ECTV is not known to directly infect CD8 T cells Although we find minimal differences in IFNα in ECTV/LCMV co-infected mice compared to mice infected with LCMV alone, we did observe significantly and consistently lower levels of systemic IFNβ in co-infected mice. Therefore, our data suggests that IFNβ, rather than IFNα, plays a larger role in supporting

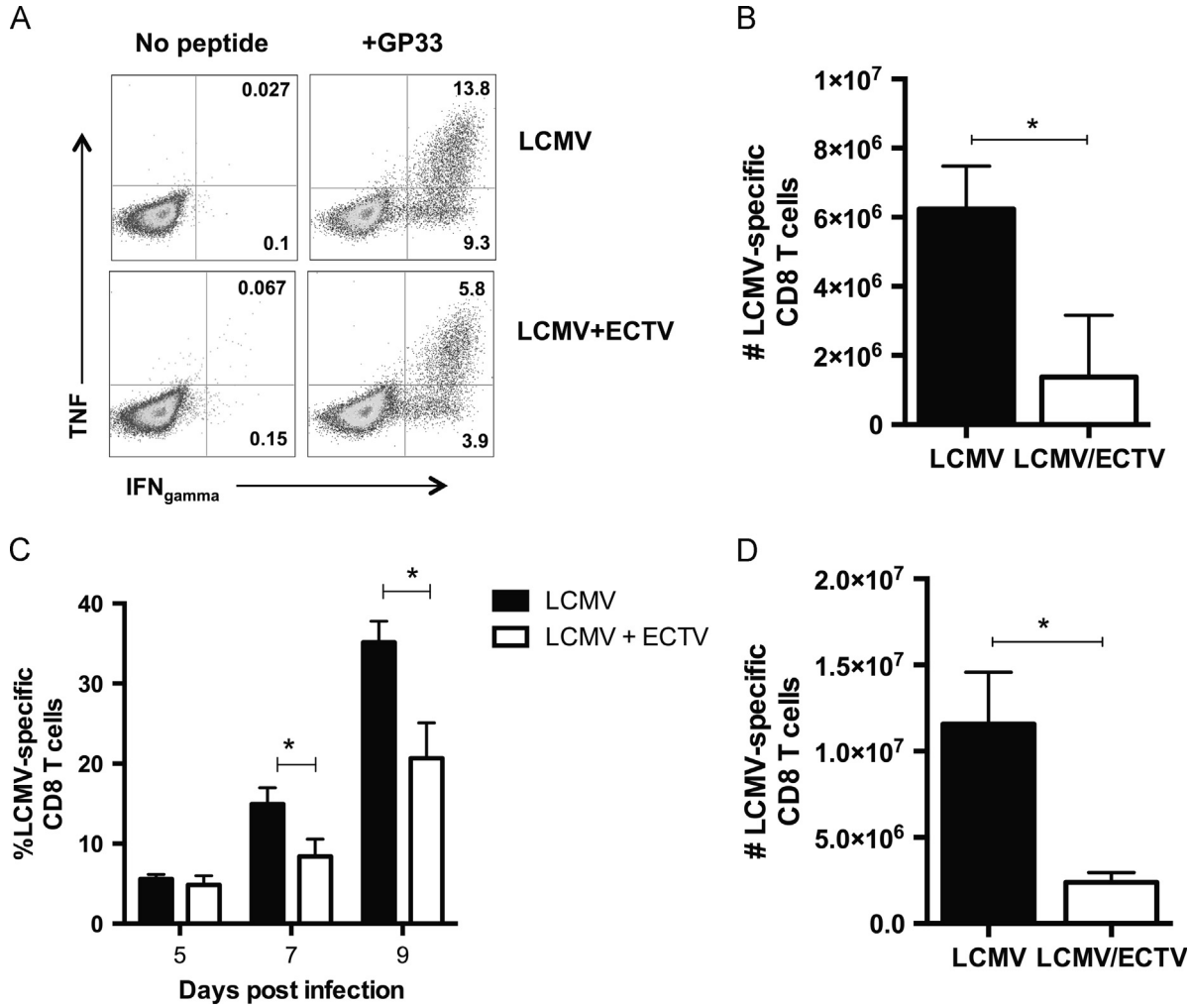

Fig. 5. Decreased IFN-I during ECTV/LCMV co-infection limits LCMV-specific CD8 T cell expansion. 6–8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. At the indicated times post infection, splenocytes were harvested, processed to single cell suspensions and stimulated for 6 h with cognate LCMV peptides prior to intracellular cytokine staining. [A] Representative FACS plots of the LCMV-specific (GP33 peptide stimulated) CD8 T cell response in the spleen at day 9 post infection of LCMV infected and ECTV/LCMV co-infected mice. [B] Mean total number of LCMV-specific (GP33 and NP396) CD8 T cells in the spleen (as measured by IFNγ production after peptide stimulation) on day 9 post infection [C] Mean LCMV-specific (GP33 and NP396) response as a percentage of total CD8 T cell population in LCMV infected and ECTV/LCMV co-infected mice at day 5, 7 and 9 post infection ($n=3$–5 mice/group). [D] 8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal via the footpad route (20 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. Mean total number of LCMV-specific (GP33 and NP396) CD8 T cells in the spleen (as measured by IFNγ production after peptide stimulation) on day 9 post infection ($n=3$–5 mice/group).

sustained CD8 T cell proliferation during LCMV infection. In addition to lower overall effector and memory T cell responses in ECTV/LCMV co-infected mice, we also show that ECTV partial suppression of Type I IFN during LCMV co-infection results in decreased memory CD8 T cell functionality and potential biasing towards an effector-memory phenotype. While we observed no difference in the ability of memory cells in either group to control subsequent LCMV infection, it has been shown that central-memory are better able to control LCMV infection compared to effector-memory cells (Kaech et al., 2002). Taken together with the decreased TNF production by these cells, our results suggest that ECTV co-infection results in a slight impairment of LCMV-specific immunity.

The importance of type I interferon to limit ECTV disease is well known (Smith and Alcami, 2002; Karupiah et al., 1993). It has previously been shown that antibody blockade of ECTV type I interferon-binding protein, C12R, during ECTV infection drastically reduces ECTV disease (Xu et al., 2012). Therefore, our results indicate that Type I IFN produced after LCMV infection may be overwhelming the ability of C12R to block signaling, mimicking antibody blockade. It is currently unknown which ECTV-encoded proteins provide the suppressive effects on Type I IFN production that result in attenuation and alteration of LCMV-specific CD8 T cell immunity. We propose that the most likely candidate in this case is the ECTV dsRNA binding protein (homolog of Vaccinia virus E3L) that would be able to limit type I interferons during co-infection (Smith and Alcami, 2002; Chang et al., 1992; Langland and Jacobs, 2002). The ECTV IFN-I binding protein has been shown to only block the action of mouse IFNα (Smith and Alcami, 2002). Our results suggest there is only a decrease in systemic IFNβ, indicating a different mechanism other than ECTV expression of an IFN-I binding protein, is responsible for limiting LCMV-specific CD8 T cell immunity. Interestingly, previous reports that showed no alteration of LCMV-specific immune responses during co-infection with the orthopoxvirus, vaccinia virus, utilized the highly attenuated "Lancy" vaccine strain that has low virulence in mice (Brenan and Zinkernagel, 1983).

The discovery of viral genomes incorporated within mammalian genomes indicates that viral families are much older than previously believed (Katzourakis and Gifford, 2010). The newly predicted timescale increases the frequency of potential viral co-divergence with hosts in order for the virus to survive (Sharp and Simmonds, 2011). In addition, hosts that are infected with multiple viruses or host that are persistently infected and undergo additional

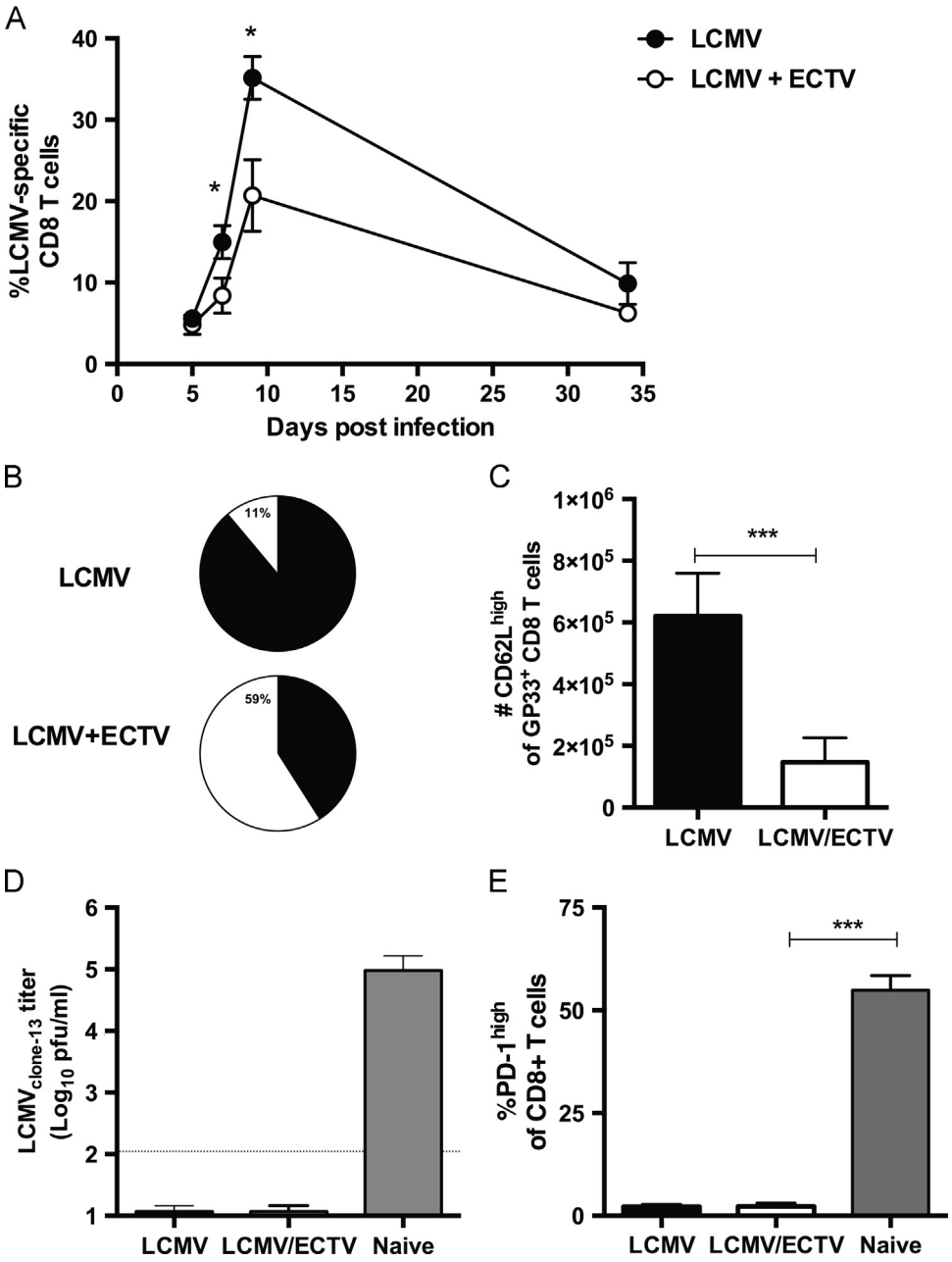

Fig. 6. Decreased CD8 T cell responses to LCMV during ECTV/LCMV co infection do not impair control of LCMV but bias T cell memory function. 6–8 week old female C57Bl/6 mice were injected with 10^5 pfu ECTV-US17-βgal ip (100 μl). Immediately following ECTV infection co-infected mice were injected with 10^5 pfu LCMV ip. LCMV immune mice were infected with only 10^5 pfu LCMV Armstrong ip. After a period of at least 35 days, surviving co-infected mice and LCMV immune mice were challenged with 2×10^6 pfu LCMV-clone 13 iv or used to enumerate LCMV CD8 T cell memory formation and function. At the indicated times post infection, splenocytes were harvested, processed to single cell suspensions and stimulated for 6 hours with cognate LCMV peptides prior to intracellular cytokine staining. [A] Kinetics of LCMV-specific (GP33 and NP396) CD8 T cell response as a percentage of total CD8 T cells in the spleen in ECTV co-infected mice and LCMV immune mice ($n=3$–5 mice/group). [B] Functional analysis of GP33 and NP396 memory CD8 T cells at day 35 post infection. White bar indicates ability to produce IFNγ, black sections indicates ability to produce both TNF and IFNγ after peptide stimulation ($n=3$–5 mice/group). [C] Number of CD62L-high, GP33-tetramer positive CD8 T cells in the spleen at day 35 post infection in LCMV infected and ECTV/LCMV co-infected mice ($n=3$–5 mice/group). [D] LCMV clone 13 viral load (pfu/ml) in the serum of LCMV clone-13 challenged mice at day 7 post infection ($n=3$–5 mice/group). [E] Percentage of PD-1^{high} GP33-tetramer positive cells at day 15 post-challenge in LCMV immune, ECTV/LCMV co-infected and naïve mice ($n=3$–5 mice/group).

infection with an unrelated virus could result in further viral co-evolution. The beneficial interplay via modulation of IFN-I between LCMV and ECTV suggest that there may have been co-evolution of ECTV and LCMV in wild mouse populations due to the potential increase in viral transmission of both viruses.

An important point that these studies raise is whether co-infection alters either ECTV or LCMV transmission in wild mouse populations. ECTV is suspected to be easily transmitted among naturally infected wild populations of mice (Esteban and Buller, 2005; Fenner, 1981). Multiple ECTV strains with varying disease severity have been isolated from outbreaks in European and North American laboratory mouse colonies (Mavian et al., 2014; Osterhaus et al., 1981; Osterrieder et al., 1994). The delayed progression of ECTV disease during LCMV co-infection that results in continued host survival may lead to greater potential for transmission of ECTV to subsequent hosts. In addition, decreased CD8 T cell immunity and function during ECTV co-infection could also result in a decreased ability to control infection, potentially leading to increased transmission of both ECTV and LCMV. Although we observed no difference in LCMV control in this experimental setting using the

acute Armstrong strain of LCMV, circulating LCMV strains in the wild also include persistent strains (Oldstone and Dixon, 1968; Becker et al., 2007; Althaus et al., 2007). Future experiments will need to address how ECTV modulates Type I IFN during infection during co-infection with persistent strains of LCMV or in established carrier mice infected at birth with the virus. However, recent data suggest that Type I IFNs produced during later time points of persistent LCMV infection actually contribute to persistence by suppressing immune responses (Teijaro et al., 2013). Therefore, our results would be consistent with a more important role for ECTV suppression of LCMV Type I IFNs during co-infection resulting in early suppression of immunity.

An important observation in these studies is that viral co-infection can alter the magnitude, function, and phenotype of CD8 T cell responses of the unrelated co-infecting virus. One potential implication of viral co-infection may be variation of T cell responses during vaccination and/or viral infection, thus the range of responses seen between individuals (Pereyra et al., 2008; Brodin et al., 2015; Li et al., 2014) may be in part explained by the context of other infections. This may be relevant for vaccination studies, using recombinant orthopoxviruses as vaccine vectors for other pathogens (Tartaglia et al., 1990), since individuals with other infections (or other vaccinations) may attenuate the effectiveness of such strategies due to innate immune modulation.

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